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Sequential In Vitro Cyclization by Cytochrome P450 Enzymes of Glycopeptide Antibiotic Precursors Bearing the X-Domain from Nonribosomal Peptide Biosynthesis

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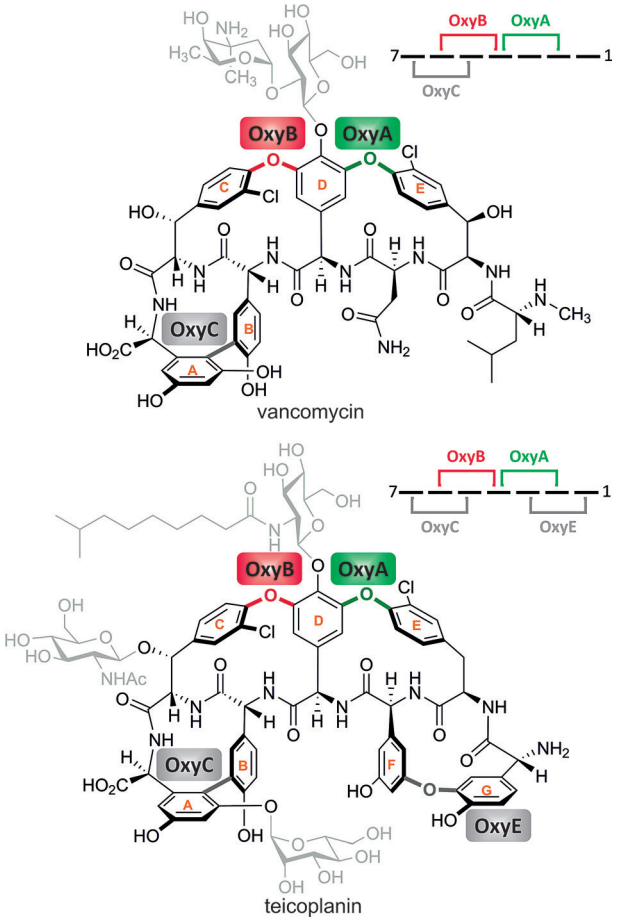
Abstract: *The biosynthesis of the glycopeptide antibiotics, which include vancomycin and teicoplanin, relies on the interplay between the peptide-producing non-ribosomal peptide synthetase (NRPS) and Cytochrome P450 enzymes (P450s) that catalyze side-chain crosslinking of the peptide. We demonstrate that sequential in vitro P450-catalyzed cyclization of peptide substrates is enabled by the use of an NRPS peptide carrier protein (PCP)-X di-domain as a P450 recruitment platform. This study reveals that whilst the precursor peptide sequence influences the installation of the second crosslink by the P450 OxyA$_{tei}$, activity is not restricted to the native teicoplanin peptide. Initial peptide cyclization is possible with teicoplanin and vancomycin OxyB homologues, and the latter displays excellent activity with all substrate combinations tested. By using non-natural X-domain substrates, bicyclization of hexapeptides was also shown, which demonstrates the utility of this method for the cyclization of varied peptide substrates in vitro.*

The Cytochrome P450 enzymes (P450s) are a superfamily of monooxygenases that are important biocatalysts in a wide range of processes.[1] P450s perform many different oxidative modifications of natural product precursors, spanning hydroxylation of C–H bonds, epoxidation of alkenes, and heteroatom oxidation.[2] Another set of impressive transformations catalyzed by P450s leads directly to alterations of the core structure of the natural product. Prominent examples are found in the biosynthesis of terpenes, steroids, and the glycopeptide antibiotics (GPAs).[3] In the case of GPAs, P450s introduce the phenolic and aryl crosslinks between the aromatic side chains of amino acid residues in the precursor peptide. These crosslinks enable the formation of the distinctive rigid three-dimensional structure that is essential for the antibacterial activity of GPAs (Scheme 1).[3f,g]

The chemical complexity of GPAs makes them challenging synthetic targets and their commercial production remains reliant upon bacterial fermentation.[3f,g] The mechanism by which Actinomycetes synthesize GPAs is based around the synthesis of the linear heptapeptide precursor by a linear, Type A non-ribosomal peptide synthetase (NRPS). The ability of the NRPS adenylation (A) domains to select and activate amino acids beyond those involved in protein biosynthesis enables the incorporation of a wide range of building blocks into non-ribosomal peptides (NRPs), such as the rigid hydroxylated phenylglycine residues in GPA precursor peptides.[4]

Following peptide biosynthesis, cyclization of the NRPS-bound GPA precursor peptide is performed by the sequential action of three or four P450s (for Type I/II and Type III/IV GPA systems, respectively) prior to final decoration of the GPA aglycone with various moieties, most commonly sugars (Scheme 1).[5] The importance of cyclization in generating active GPA antibiotics makes this process the key biosyn-



Scheme 1. Structures of the Type I and Type IV glycopeptide antibiotics vancomycin and teicoplanin, highlighting initial side-chain crosslinking reactions catalyzed by the Cytochrome P450 enzymes OxyB (red) and OxyA (green).

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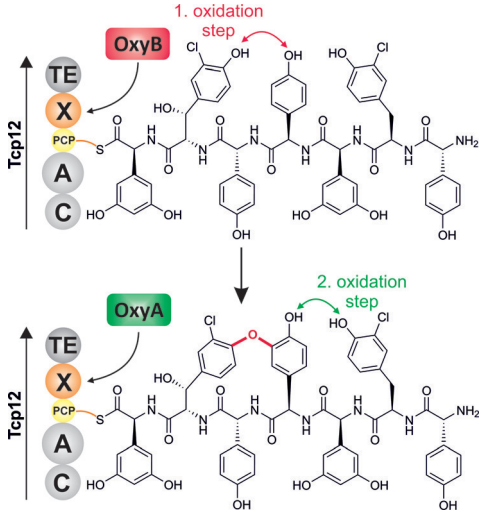
thetic step and underpins the elegant simplicity of GPA biosynthesis.

Recently, we demonstrated that P450 recruitment to the NRPS-bound peptide in GPA biosynthesis relies not upon the carrier protein[6]—as for the majority of such systems[7]—but rather upon a specific conserved recruitment domain in the terminal NRPS module: the X-domain (Scheme 2).[8] This



Scheme 2. Initial cyclization of the teicoplanin precursor heptapeptide by OxyB and OxyA involves the NRPS X-domain recruitment platform.

domain is a modified condensation (C) domain that provides the platform to recruit all essential P450s to the adjacent peptide carrier protein (PCP)-bound peptide.[8] A natural fusion of the X-domain and the peptide-bound PCP domain allowed sequential P450 cyclization activity to be detected in vitro for the first time. However, the effect of this domain on the selectivity for these substrates exhibited by the crosslinking P450s (Oxy enzymes) was unknown. Herein, we demonstrate that an in vitro cascade of the first two GPA oxygenase enzymes ($OxyB_{van/tei}$ and $OxyA_{tei}$) can generate bicyclic products from a range of precursor peptides when presented by PCPs naturally fused to the X-domain.

To assess the ability of an OxyB/A cascade to generate bicyclic GPA peptides, installation of the C-*O*-D ring for heptapeptide-bound PCP-X constructs by OxyB was characterized. Previous results had indicated effective cyclization of teicoplanin- and vancomycin-like substrate peptides by OxyB enzymes from related (Type IV and Type I) GPA biosynthetic systems,[8] but the effect of altering the peptide sequence in such experiments was unknown. Therefore, heptapeptide–CoA conjugates (**1–4**, Figure 1) mimicking structurally diverse GPAs were synthesized by using solid-phase peptide synthesis (see the Supporting Information).[9] After loading onto PCP-X di-domain constructs, the peptides served as substrates for the Oxy enzymes; since racemization of Hpg_7 cannot be prevented during synthesis, peptides **1–4** were used as a pair of epimers (see the Supporting Information). We considered the additional complexity of analyzing product mixtures resulting from coupled enzymatic transformations and identified methylamine[10] as an ideal reagent for

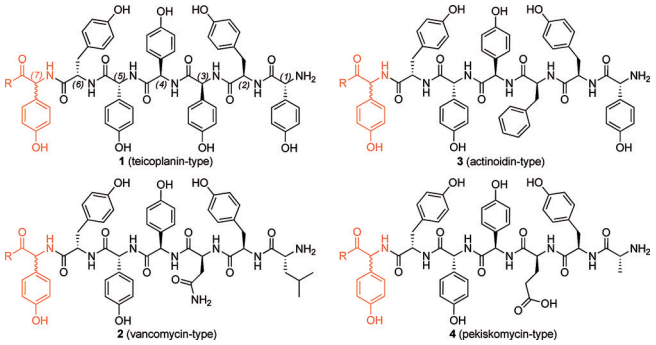
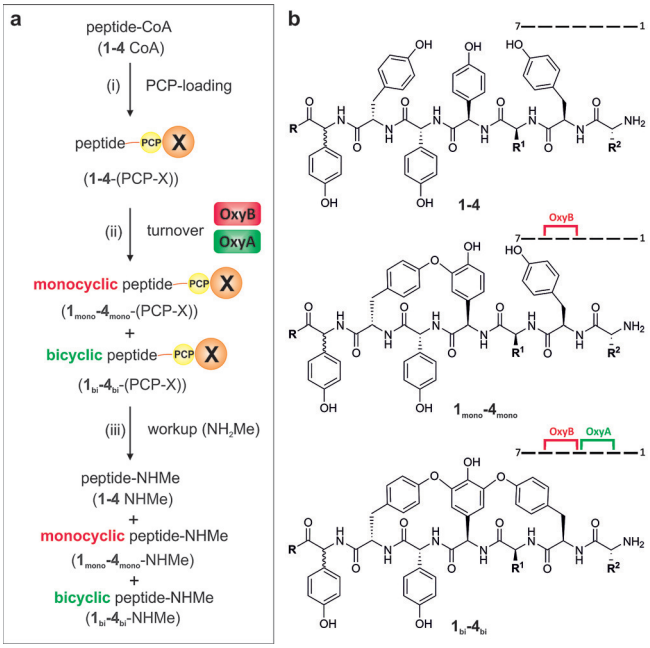


Figure 1. Structures of the heptapeptides **1**–**4** and hexapeptides **1a**–**4a** (which lack the 7th residue (orange)) used in this study (R = CoA, PCP-X, NHMe).

thioester cleavage upon workup (Figure S8 in the Supporting Information).

Initial experiments were performed to find the best NRPS constructs to act as carriers for peptide substrates (Scheme 3). Both teicoplanin ($PCP\text{-}X_{tei}$)[11] and vancomycin ($PCP\text{-}X_{van}$)[12] constructs could serve as delivery platforms for peptides to either OxyB homologue, although $OxyB_{tei}$ activity was reduced when using $PCP\text{-}X_{van}$. Since native PAGE analysis indicated complex formation in all cases (Figure S5 in the Supporting Information), the observed differences in OxyB activity must be due to subtle alterations in peptide presentation.

Next, we examined the efficiency of C-*O*-D ring installation by $OxyB_{van}$/$OxyB_{tei}$ in **1–4** bound to $PCP\text{-}X_{tei}$. We observed significant activity of $OxyB_{tei}$ with all of the peptides (Table 1 and Figure S9); non-teicoplanin-like peptides **3**/**4**-



Scheme 3. a) In vitro assay sequence (PCP loading, turnover, and workup. b) Structures of linear (**1–4**), monocyclic ($\mathbf{1}_{mono}$–$\mathbf{4}_{mono}$), and bicyclic ($\mathbf{1}_{bi}$–$\mathbf{4}_{bi}$) peptides. R = CoA, PCP-X, or NHMe, as indicated in panel (a), and R^1 and R^2 are defined in Figure 1.

Table 1: Binding and oxidation of **1–4**-(PCP-X)$_{tei}$ by OxyB homologues.

Oxy	Substrate	Amplitude	Spin-state shift [%]	k_D [μM]	Turnover [%][a]
B_{tei}	**1**-(PCP-X)$_{tei}$	0.16 ± 0.01	48	0.9 ± 0.2	65.7 ± 1.4
	2-(PCP-X)$_{tei}$	0.02 ± 0.01	6	0.4 ± 0.3	38.4 ± 2.8
	3-(PCP-X)$_{tei}$	0.02 ± 0.01	7	0.3 ± 0.2	71.3 ± 1.6
	4-(PCP-X)$_{tei}$	0.04 ± 0.01	12	0.3 ± 0.1	77.5 ± 0.7
B_{van}	**1**-(PCP-X)$_{tei}$	0.07 ± 0.01	48	ND[b]	72.3 ± 4.9
	2-(PCP-X)$_{tei}$	0.09 ± 0.01	57	ND[b]	94.5 ± 1.8
	3-(PCP-X)$_{tei}$	0.08 ± 0.01	52	ND[b]	88.8 ± 0.9
	4-(PCP-X)$_{tei}$	0.08 ± 0.01	52	ND[b]	86.4 ± 0.4

[a] Triplicate experiments. [b] Active-site titration, saturation at 0.9 μM substrate concentration.

(PCP-X)$_{tei}$ were cyclized to more than 70%. This is a marked improvement over previous results for PCP-bound hexapeptides, where OxyB$_{tei}$ activity was only observed for **1a**.[13] OxyB$_{van}$ displays excellent activity against **1–4** and gives results comparable to those with PCP-bound hexapeptides.[9a,14] Given the differences in behavior of the OxyB homologues, we investigated whether substrate binding correlated with oxidation efficiency. For OxyB$_{van}$, this appears to be the case: all substrates bind in the manner of an active site titration and stimulate a significant spin-state shift of the P450 (Table 1 and Figure 2). For OxyB$_{tei}$, the dissociation constant of all substrates is higher and the level of spin-state shift elicited varies significantly (Table 1 and Figure 2), thus indicating that whilst binding of the natural substrate **1**-(PCP-X)$_{tei}$ is best at triggering the spin-state shift of OxyB$_{tei}$, this is not in and of itself an essential requirement for activity. Indeed, the differences between the homologues in terms of correlation between spin-state shift and activity suggest subtle differences in the binding of the peptide substrates, which can most likely be traced back to interaction of the P450 with the PCP-domain.

Characterization of the OxyA homologues indicated that OxyA$_{tei}$ is catalytically competent and maintains the thiolate-ligated heme state of an active P450, whilst OxyA$_{van}$ largely exists in the incompetent P420 state (Figure S6). This was confirmed in a coupled assay with OxyB$_{van}$ and the cognate substrate peptides (see Figure 2 and Table 2). OxyA$_{tei}$ converted approximately 40% of **1**$_{mono}$-(PCP-X)$_{tei}$ into the bicyclic product **1**$_{bi}$-(PCP-X)$_{tei}$, whilst OxyA$_{van}$ activity was minimal. We also tested conditions for such a coupled assay by altering the concentrations of OxyB$_{tei}$ and OxyA$_{tei}$. The results show that OxyB concentration can be reduced eightfold with no drop in activity, but OxyA$_{tei}$ activity was most efficient under the original conditions tested (Figure S9).

We then characterized the selectivity of OxyA$_{tei}$ for different peptide substrates (Table 2 and Figure S10). The results indicate that there is a distinct preference for **3** in OxyA-catalyzed cyclization and that the native structure **1** is well accepted. These turnover results are independent of the OxyB homologue used to generate the monocyclic OxyA substrate, thus indicating that the sequence of the peptide is the agent governing the level of activity observed. The structural differences in **1–4** center around the site of the D-*O*-E crosslink installed by OxyA; the effects of different peptide amino acid substitutions would thus be situated close to the enzyme active site and could readily influence enzymatic activity. Given that the two best peptide substrates for OxyA both contain an Hpg$_1$ residue (**1**/**3**$_{mono}$-(PCP-X$_{tei}$)) this would appear to be important in correctly orienting the peptide in the active site. The differences in cyclization for **1**$_{mono}$-(PCP-X$_{tei}$) and **3**$_{mono}$-(PCP-X$_{tei}$) must then center on the different residue present at position three of the peptide. This implies that the orientation of both amino acid side chains

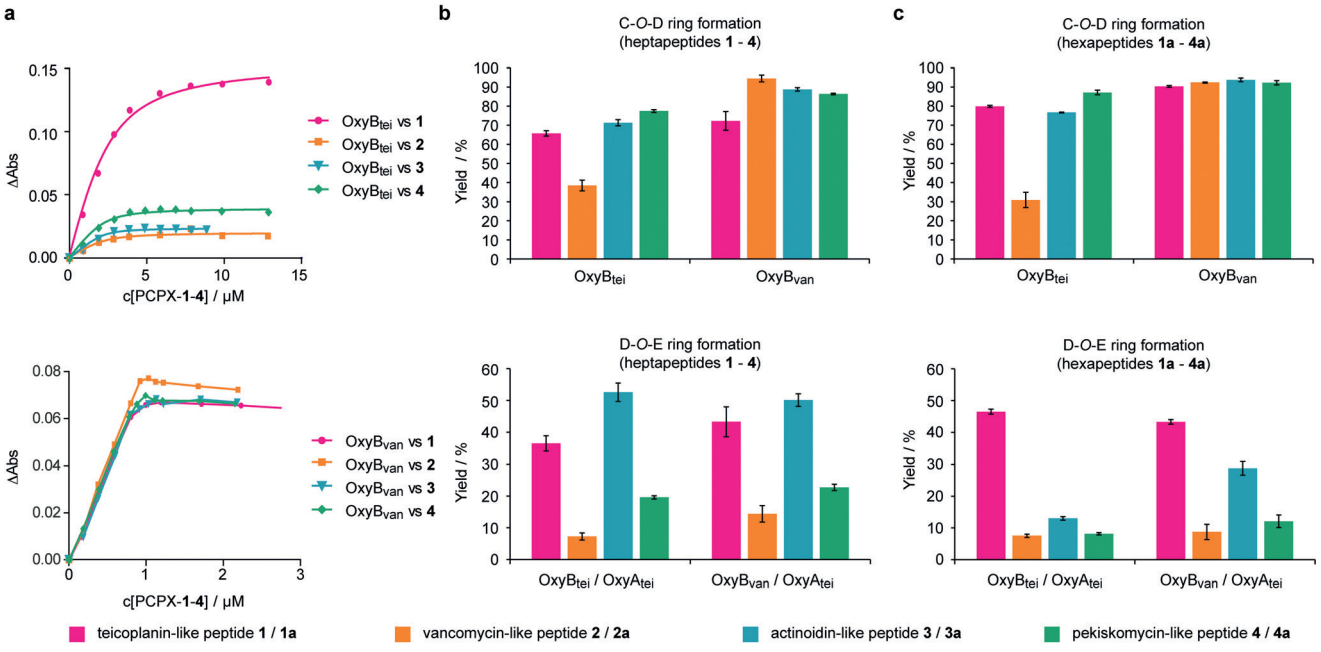


Figure 2. a) Binding of **1–4**-(PCP-X$_{tei}$) to OxyB homologues b, c) Quantification of the cyclization of peptide substrates **1–4**-(PCP-X) (b) and **1a–4a**-(PCP-X) (c) catalyzed by OxyB and OxyA homologues (upper and lower panels, respectively).

Table 2: Oxidation of **1**–**4**-(PCP-X)$_{tei}$ in a coupled assay with OxyB and OxyA homologues.

OxyB	OxyA	Substrate	C-*O*-D formation [%][a]	D-*O*-E formation [%][a]
B_{tei}	A_{tei}	**1**-(PCP-X)$_{tei}$	67.5 ± 4.7	36.5 ± 2.4
		2-(PCP-X)$_{tei}$	52.6 ± 3.1	7.3 ± 1.1
		3-(PCP-X)$_{tei}$	70.9 ± 6.7	52.6 ± 2.9
		4-(PCP-X)$_{tei}$	75.5 ± 0.8	19.6 ± 0.5
B_{van}	A_{tei}	**1**-(PCP-X)$_{tei}$	69.0 ± 2.7	43.3 ± 4.7
		2-(PCP-X)$_{tei}$	92.8 ± 0.3	14.4 ± 2.6
		3-(PCP-X)$_{tei}$	88.2 ± 0.6	50.1 ± 2.0
		4-(PCP-X)$_{tei}$	88.7 ± 0.4	22.7 ± 1.0
B_{van}	A_{van}	**2**-(PCP-X)$_{tei}$	73.7 ± 5.6	1.1 ± 1.5

[a] Triplicate experiments.

involved in formation of the crosslink must be carefully controlled to allow effective Oxy activity.

We further examined the effect of peptide length on the efficiency of OxyB and OxyA by utilizing hexapeptides related to **1**–**4** (**1a**–**4a**, Figure 1). The results obtained for both OxyB homologues indicate that processing of **1a**–**4a** is at least as efficient as that of **1**–**4** and often higher: levels of $OxyB_{van}$ cyclization were now above 90% for all substrates (Table 3). $OxyA_{tei}$ activity against **1a**$_{mono}$-(PCP-X)$_{tei}$ remained high, whilst **3**/**4a**$_{mono}$-(PCP-X)$_{tei}$ were poorer substrates than their heptapeptide equivalents, thus indicating less flexibility in the oxidation of hexapeptide substrates by $OxyA_{tei}$.

Taken together, these results indicate that OxyA activity is enabled by the X-domain and $OxyB_{tei}$ activity is enhanced. This supports the general role of the X-domain in P450 recruitment during GPA maturation. The differences in activity between the OxyB homologues indicate that the ability of each Oxy enzyme to form a productive conformation with the PCP-bound peptide substrate determines the level of product formed, with $OxyB_{van}$ able to populate this conformation to a larger extent than $OxyB_{tei}$. Effective cyclization of heptapeptides by OxyB enzymes, even in the case of $OxyB_{van}$, appears to be a challenging reaction and requires the presence of the X-domain to assist in correctly positioning the PCP-bound substrate. With regards to $OxyA_{tei}$, the amino acids directly in the environment of the D-*O*-E ring linkage influence the efficiency of catalysis. However, given that hexa- and heptapeptides of all of the GPA classes tested are converted into bicyclic products, it would appear that OxyA enzymes are not confined to their native substrates. The marked differences in the in vitro selectivity and activity shown by the OxyB and OxyA enzymes from the teicoplanin and vancomycin GPA systems highlight the general importance of screening homologues to identify those most suitable for assembling a biomimetic cascade for GPA biosynthesis, a necessity that also applies to the glycosyltransferases involved in GPA biosynthesis.[4a]

Table 3: Oxidation of **1**–**4a**-(PCP-X)$_{tei/van}$ by OxyB alone and in a coupled assay with OxyB and OxyA homologues.

OxyB	OxyA	Substrate	C-*O*-D formation [%][a]	D-*O*-E formation [%][a]
B_{tei}	–	**1a**-(PCP-X)$_{tei}$	80.0 ± 0.5	–
		2a-(PCP-X)$_{van}$	31.0 ± 4.0	–
		3a-(PCP-X)$_{tei}$	76.8 ± 0.2	–
		4a-(PCP-X)$_{tei}$	87.3 ± 1.2	–
B_{van}	–	**1a**-(PCP-X)$_{tei}$	90.5 ± 0.4	–
		2a-(PCP-X)$_{van}$	92.5 ± 0.3	–
		3a-(PCP-X)$_{tei}$	93.9 ± 1.0	–
		4a-(PCP-X)$_{tei}$	92.4 ± 1.1	–
B_{tei}	A_{tei}	**1a**-(PCP-X)$_{tei}$	89.5 ± 1.0	46.6 ± 0.8
		2a-(PCP-X)$_{van}$	34.0 ± 2.3	7.6 ± 0.5
		3a-(PCP-X)$_{tei}$	91.8 ± 0.6	13.1 ± 0.6
		4a-(PCP-X)$_{tei}$	87.8 ± 1.5	8.2 ± 0.3
B_{van}	A_{tei}	**1a**-(PCP-X)$_{tei}$	77.9 ± 1.6	43.4 ± 4.7
		2a-(PCP-X)$_{van}$	92.5 ± 2.3	8.8 ± 2.4
		3a-(PCP-X)$_{tei}$	86.5 ± 7.5	28.8 ± 2.2
		4a-(PCP-X)$_{tei}$	92.7 ± 0.4	12.2 ± 2.0

[a] Triplicate experiments.

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